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(54) Title: *IN PLANTA* TRANSFORMATION OF PLANTS

(57) Abstract

Methods of delivering isolated DNA to target cells of whole plants are disclosed wherein *Agrobacterium* compositions are delivered directly to plants for transformation obviating the need to regenerate transformed plants from tissue culture or explants.

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IN PLANTA TRANSFORMATION OF PLANTS

RELATED APPLICATION

This application claims the benefit of U.S. Provisional Application No. 60/058,718, filed September 12, 19987, the contents of which are incorporated herein by reference in their entirety.

BACKGROUND OF THE INVENTION

It has generally been assumed that the range of species amenable to successful *in planta* transformation of plants is very limited, making this procedure unavailable for most plants, including elite varieties of crop plants. Only one dicotyledonous species, *e.g.*, *Arabidopsis thaliana*, which is easily transformed by most other methods of gene transfer, has been successfully transformed by an *in planta* procedure.

The ability to transform important agricultural and horticultural varieties of plants using *in planta* techniques would substantially reduce the time necessary to produce transgenic plants carrying valuable new traits. In addition, expensive and lengthy tissue culture phases required for regenerating new plants from cells transformed by other methods can be bypassed.

SUMMARY OF THE INVENTION

This invention relates to a process for the incorporation of isolated DNA into chromosomes of higher plants, *e.g.*, *Lactuca sativa* and *Brassica napus*. Reliable and efficient methods of transformation have been developed for these two plant species. These techniques present

substantial improvements over the *in planta* transformation method previously reported for *Arabidopsis thaliana* (Chang *et al.*, 1990; Katavic *et al.*, 1994), and are designed to optimize frequency of transformation. Preferred techniques of the invention for obtaining the desired transformed lettuce and *Brassica* plants include the use of *Agrobacterium tumefaciens* strains (having appropriate virulences), or other abiotic means (such as chemical pre-treatment or co-treatment, electroporation, biolistics, etc.), and use of efficient promoters. An alternate abiotic preparation may include the use of chemically synthesized compounds which solubilize the plant cell walls, applied either before or with the DNA solution to be transformed into the plant.

In one embodiment, the transformation procedure involves severing apical shoots at their bases, inoculating the wound sites of the bases with droplets of an *Agrobacterium tumefaciens* (or abiotic compound with or prior to applying the DNA), and generating new shoots *in planta* from the severed sites. A preferred embodiment involves soaking a small plug of rock wool with the *A. tumefaciens* culture (or other abiotic preparation containing DNA) and applying the plug to the wound site. Another variation of this method involves applying the abiotic preparation (with or prior to applying the DNA) to an uncut shoot (*i.e.* no prior wounding).

This invention also relates to transformed higher plants which contain novel nucleotide constructions capable of stable expression. Seed and progeny of these plants are included in this invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a diagram of the CAMV-Rbcs-Cab transgene

for introduction into plants.

Figure 2 is a diagram of the CAMV-Bce44B-NOS transgene for introduction into plants.

The foregoing and other objects, features and advantages of the invention will be apparent from the following more particular description of preferred embodiments of the invention, as illustrated in the accompanying drawings in which like reference characters refer to the same parts throughout the different views. The drawings are not necessarily to scale, emphasis instead being placed upon illustrating the principles of the invention.

DETAILED DESCRIPTION OF THE INVENTION

Gene manipulation techniques are continuously being developed as useful tools for improving crops. Genetic transformation is used to broaden the genetic base of germplasm available for conventional breeding, and to reduce the time required to introduce single gene traits into crop plants. Further, genetic transformation of plants permits the production and harvesting of foreign proteins and other products from plant cells.

The essential requirements of a gene transfer system for production of transgenic plants are: (a) availability of a target tissue including cells competent for plant regeneration, (b) a method to deliver DNA into those regenerable cells, and (c) a procedure to select and regenerate transformed plants at a satisfactory frequency.

Gene transfer in higher plants is traditionally based on the use of *in vitro* cultured cells or tissues in which DNA is integrated into the nuclear genome by various methods. Physical methods include electroporation (Fromm *et*

al., 1982), microinjection (Reich *et al.*, 1986), biolistics (Klein *et al.*, 1987), and ultrasonication (Li-Jian *et al.*, 1991). Over the past few years a number of *Agrobacterium*-mediated transformation systems have also been developed (for review, see Zambryski, 1992). Although the *Agrobacterium*-mediated transformation systems can be used to produce large numbers of transformants, they all rely on tissue culture techniques. Tissue culture is labor-intensive and can be difficult to master. In addition, even under optimal transformation and regeneration conditions, tissue culture can result in somaclonal variation or morphological abnormalities (van den Bulk *et al.*, 1990; Evan and Sharps, 1986; Larkin and Scowcroft, 1982), changes in chromosome number and loss of fertility (Scholl *et al.*, 1981). One of the simplest available plant transformation systems involves infiltration of *Agrobacterium* cells into *Arabidopsis* plants before flowering, and direct selection for transformants in the resulting seedling populations (Chang et al., 1994; Bechtold et al., 1993).

Of the higher plants, lettuce, flax and *Brassica* species are three important, extremely valuable vegetable and field crop species which are amenable to manipulation by molecular techniques. Others include, but are not limited to, corn and wheat. Modification of higher plants using genetic engineering techniques can facilitate the rapid development of new varieties with traits such as herbicide resistance, disease resistance, or seed quality improvement. Of greatest interest is the use of introduced genes constructed with promoters that can confer specific gene expression in the transformants, such as constitutive, inducible, stage-specific, or tissue-specific expression. Infection by *Agrobacterium spp.* has been the best method

available for DNA transfer to tissue explants. Lettuce can be transformed routinely using *Agrobacterium tumefaciens* (Michelmore *et al.*, 1987; Enomoto *et al.*, 1990; Curtis *et al.*, 1994).

This invention relates to methods for the incorporation of isolated DNA into chromosomes of higher plants, both monocots and dicots, including corn, wheat, flax, lettuce and *Brassica napus*, and obviates the time-consuming and labor intensive *in vitro* regeneration step. The term "isolated DNA" refers to DNA which is isolated from organisms; that is, it is separated away from the nucleic acids of genomic or cellular DNA or DNA as it exists in a mixture of nucleic acids. The isolated DNA can include foreign DNA, which is DNA not naturally-occurring in the species into which it is incorporated by the methods described herein. It can also include altered, recombinant or synthetic DNA. By altered, it is meant that the isolated DNA is changed from its original sequence by the deletion, addition or substitution of one or more nucleotides.

The transformation protocol is an improvement of the *in planta* transformation method first described by Chang *et al.* (1990) and Katavic *et al.* (1994) for *Arabidopsis thaliana* in that it is applicable to all species of plants, including monocots which are difficult to transform. Thus, elite varieties of crop and horticultural plants can be transformed and transgenic seed obtained within short periods of time without the expense of long tissue regeneration periods. Further, the methods of this invention lead to stably transformed plants. Regenerated plants do not appear to exhibit somaclonal variation or morphological abnormality or reduced fertility, which constitute problems associated with tissue culture

techniques. Examples of transformed lettuce, *Brassica napus*, flax, tomato, soybean basil, pea, garden bean, alfalfa, petunia, corn and wheat plants are provided. These are crop and ornamental plants which have heretofore not been transformed by an *in planta* transformation system.

These methods also provide the means through which plant species that do not form tissue culture can be transformed. For example, tissue cultures cannot be obtained from *Impatiens* sp., thus regeneration of whole transformed plants is not possible so that traditional transformation procedures cannot be used on this species. The *in planta* transformation methods of this invention can be used to transform such species.

The novel methods of this invention involve the introduction of nucleotide constructs into cells of higher plants wherein the resultant transformed cells express one or more genes present in the construct so as to provide at least one novel property for the plants, particularly a phenotypic property. Any gene in which expression of its product can be induced in plants can be used in the practice of this invention. The expression of a plant gene which exists in double-stranded form involves transcription of messenger RNA (mRNA) from one strand of the DNA by RNA polymerase enzyme, and the subsequent processing of the mRNA primary transcript inside the nucleus. This processing involves a 3' non-translated region which causes addition of polyadenylate nucleotides to the 3' end of the RNA.

Transcription of DNA into mRNA is regulated by a region of DNA usually referred to as the "promoter". The promoter region contains a sequence of bases that signals RNA polymerase to associate with the DNA, and to initiate the transcription of mRNA using one of the DNA strands as a template to make a corresponding strand of RNA. A number of

promoters which are active in plant cells have been described in the literature and are suitable for the methods of this invention. These include the nopaline synthase (NOS) and octopine synthase (OCS) promoters, the cauliflower mosaic virus (CaMV) 19S and 35S promoters, the light-inducible promoter from the small subunit of ribulose bis-phosphate carboxylase (ssRUBISCO, a very abundant plant polypeptide), promoters of genes encoding glycoproteins, and others. Promoters which are known or are found to cause transcription of RNA in plant cells can be used in the present invention. Such promoters may be obtained from plants or plant viruses. Those of skill in the art will recognize that any construct used should include a transcriptional initiation region which efficiently functions in the plant species to be transformed.

Various antibiotics can be used as selectable markers in the production of transgenic plants. In higher plant transformation, the most commonly used is the neomycin phosphotransferase gene (*nptII*) from transposon Tn5 (Bevan *et al.*, 1983) which confers resistance towards kanamycin, neomycin, gentamicin and paromomycin. Ideally a selectable marker should by controlled by a constitutive promoter such as the 35S CaMV promoter. As most promoters demonstrate tissue specificity, the promoter selected should be one that gives the highest expression level for the selectable marker gene.

A number of process steps are involved in providing for efficient frequency of *Agrobacterium* transformation. The initial stage is the selection of an *Agrobacterium tumefaciens* strain which provides for efficient transformation of the desired plant species. A number of strains of *Agrobacterium tumefaciens* are capable of transferring genetic material to plant species. For

example, successful transformation can be attained by using *Agrobacterium tumefaciens* strains LBA4404 and GV3101 for lettuce and *Brassica* species. These strains have good host specificity and a rapid growth rate. The *Agrobacterium* to be employed as the gene transfer system is conveniently transformed with a wide-host range plasmid that can shuttle DNA from *E. coli* into *Agrobacterium*.

The methods of this invention are applicable to any plant species which provides *in planta* cells or tissues capable of regenerating into an intact shoot or plant. Such plants include the angiosperms, including both monocots and dicots. Further, the nonvascular plants, such as the ferns, bryophytes and mosses can be transformed by these methods. Preferred plants include monocots such as corn, rice, sugar cane, sorghum, barley, wheat, oats, rye, and the like. Preferred dicots include *Brassica* sp., lettuce, flax, soybean, sunflower, potato, petunia, alfalfa, bean, pea, basil, Impatiens, rose, tomato, citrus species, and the like.

Once the expression construct has been prepared and analyzed to insure it has the proper sequence, it can be used for introduction into the plant. The methods involve wounding the plant to facilitate introduction of the DNA by techniques such as, but not limited to, dissection, cutting, puncturing, injecting, and/or micro-particle bombardment. Wounding can also be accomplished by compounds which attack or dissolve the cell walls and penetrate cuticles. Chemically synthesized and natural compounds exist which can act on a plant's cell wall, degrading or dissolving it to a degree such that DNA solutions can then enter the plant and essentially transform it. Examples of such abiotic compounds are phomalide, soaps, detergents, and the like. Other abiotic systems would include any physical means (as

opposed to biological) of transformation, such as electroporation, biolistics, etc. Wounding may be enhanced by combining two or more of these techniques.

After wounding, the exposed cells or tissue can be subjected to *Agrobacterium* sp. treatment with or without some prior incubation period. Other means of applying DNA can be carried out in the same manner. In one preferred embodiment of this invention, the cells or tissues to be transformed can be treated with at least one compound which induces the virulence of the *Agrobacterium*. Suitable compounds include, but are not limited to, acetosyringone or other plant extracts for inducing *Agrobacterium* virulence. Other additives can be applied to enhance successful infection, including opines such as octapine, nopaline, leucinopine and the like.

The *Agrobacterium* strain will include on a plasmid the DNA construct, which is destined to be transferred to the plant. If using an abiotic system of DNA transfer, the naked DNA can be applied with or after the abiotic compound in order to enter the plant. To ensure successful transformation of as many cells as possible, it is important to maintain the isolated DNA in good condition and in contact with the plant cells over a period of several days. As a result of such a transfer, the DNA construct will normally be present in all or substantially all of the cells of the plant after transformation, although expression may be limited to particular cells or particular stages in the development of the plant. The DNA construct will include transcriptional and translational initiation and termination signals, with the initiation signals 5' to the gene of interest and the termination signals 3' to the gene of interest in the direction of transcription.

Thus, this invention relates to a method of delivering

isolated DNA to the cells of a plant comprising the steps of a) wounding the plant; and b) applying the isolated DNA to the wound under conditions whereby additional DNA can be applied to the wound site over a period of two or more days to enhance transformation efficiency. This confers the advantage of being able to deliver the DNA to the same target cells over a long period of time instead of randomly rewounding the plant and applying the DNA to different sites.

In a particular embodiment, a method for transforming a plant with isolated DNA can comprise preparing a transformation competent *Agrobacterium* sp. containing a Ti plasmid, wherein the DNA is contained in the plasmid and maintaining the *Agrobacterium* sp. in a cultivation medium. The plant is then wounded by any of the means discussed *supra* and the *Agrobacterium* sp. in the cultivation medium is applied to the wound with a carrier which also assists in the entry of the *Agrobacterium* sp. and which further permits the *Agrobacterium* sp. in culture solution to be replaced with additional fresh *Agrobacterium* sp.. The *Agrobacterium* sp. is maintained in the wound until the DNA is introduced into the plant and transformation of plant cells occurs. Selection is then carried out for growing portions of the plant which are transformed, thereby producing a transformed plant or portion of a plant.

One particular transformation procedure involves severing apical shoots at their bases, inoculation with droplets of an *Agrobacterium tumefaciens* (or abiotic compound with or prior to applying the DNA) at the severed sites, and *in planta* generation of shoots from the severed sites. A preferred embodiment of this procedure involves soaking a small plug of rock wool (or other abrasive

material which can hold the solution) with the *A. tumefaciens* culture (or other abiotic preparation containing DNA) and applying the plug to the wound site. The rock wool confers several unique advantages over application of the culture in a naked droplet. The rock wool can be pushed into the tissue and its abrasiveness used to further wound cells. Additionally, it provides a convenient marker to show where the culture was applied so that fresh solution can be added over several days to the same spot, increasing the exposure to the *Agrobacterium*, thus increasing the probability of successful entry and transformation of cells. The cells can therefore have a long exposure to an *Agrobacterium* culture which is always fresh and in which the *Agrobacterium* cells are multiplying.

Another embodiment involves injecting plants, for example, at the base of the stalk of a monocot, so that the bacterial suspension is applied just above where the stalk becomes hollow in older plants. This maintains the culture at the growing points and young tissues.

An alternative embodiment of this method involves applying the abiotic or *Agrobacterium* preparation (with or prior to applying the DNA) to an uncut shoot (*i.e.*, no prior wounding). This procedure (with or without rock wool) also leads to stably transformed plants. Regenerated plants do not exhibit somaclonal variation or morphological abnormality or reduced fertility, which are problems associated with tissue culture techniques. This procedure can be particularly useful for monocots.

In the techniques described above, incubation and/or application of DNA transfer and associated materials, included *Agrobacterium* sp. cultures is done at a suitable temperature for a suitable time period. In most instances,

the transfer (or incubation) will be carried out over one day to several weeks at room temperature. In some instances, plants will not be watered following application of the *Agrobacterium* culture to improve infiltration of the bacterial suspension. Secondary shoots can be removed after several days and fresh inoculum applied to the wound site, if applicable, or reinjected, or leaf tubes refilled. These inoculations can be repeated.

Putative transformed plants are subsequently analyzed to confirm that integration of isolated DNA has actually occurred, and that the integration is stable through meiosis. In addition, the gene should be expressed to a satisfactory degree. The primary transformed plants are grown to maturity and T2 seeds are collected. The integration and copy number is tested by DNA Southern hybridization, transcription by RNA Northern hybridization and finally the stability and segregation in selfed progeny of primary transformants and agronomic characters are evaluated.

In screening for transformants the selectable marker neomycin phosphotransferase *nptII*, associated with the expression construct, can be employed to provide antibiotic resistance of transformed seedlings to kanamycin. Genomic integration of foreign DNA is ideally determined by Southern hybridization (Southern, 1975). Genomic DNA is isolated, digested with endonucleases, separated electrophoretically, transferred and fixed to a filter. A labeled probe of known sequence is then hybridized to homologous sequences in the digested genomic DNA, confirming the presence of the gene and determining the copy number. Transcription of the inserted DNA into RNA is confirmed with isolated total RNA submitted to Northern hybridization (Alwine *et al.*, 1979). Self pollination of fertile transformants assesses whether

integration is stable through meiosis, and segregation of the inserted gene in the seedling progenies gives information about copy number.

Using the *in planta* transformation system, at least 50 transformed lettuce plants and at least 4 transformed *Brassica napus* plants have been obtained. Preliminary screening of flax plants shows a high percentage (over 10% by PCR analysis) of plants containing the transgene. These methods have also been applied to corn, wheat, tomato, soybean, basil, pea, bean, alfalfa, petunia and a number of other flowering plants and vegetable species.

Transformed plants produced by these methods produce seeds in short periods of time compared to other methods where transgenic plants must be regenerated from tissue culture. In some instances where no tissue culture can be produced from particular plant species, this can be the only method of obtaining transgenic plants and seed. The seed can be used to produce additional transgenic plants. Further portions of these plants can be used to propagate additional plants, for tissue culture, or to study the effects of the transgene. Portions of these plants can include leaves, stems, roots, flowers, seeds, meristems, tissue culture, protoplasts, embryos, pollen, ovules, explants, cells, and the like.

Example 1. Plant material

Seeds of *Lactuca sativa* L. cv. Bautista (romaine lettuce), *Lactuca sativa* L. cv. Bella Green (head lettuce), *Brassica napus* L. cv. Quantum, and *Linum usitatissimum* (flax) were planted in flats of sterile #1 Sunshine Mix soil and grown in a growth chamber at 21°C, 80% humidity, 65 $\mu E\ m^2/s$ light intensity, and 16 hour photoperiod. Three weeks post-germination seedlings were transplanted into 6

inch pots of #1 soil and one week later were moved to the greenhouse (16 hour photoperiod, 800-1000 μE m^2/s light intensity, 24±°C). After two weeks, the plants were ready for the first inoculation with *Agrobacterium*, which was followed by three subsequent inoculations at one week intervals. Essentially the same procedures were used to grow and inoculate tomato, soybean, basil, pea, garden bean, alfalfa, and petunia plants.

Example 2. Vector construction

Construction of a CAMV-Rbcs-Cab transgene for transformation into plants.

Transgenic plants (*Lactuca sativa* cv. Bella Green and cv. Bautista) were produced. The coding portion of the gene construct was a fusion of a DNA sequence encoding the mature portion of the type I LhcIIb Cab protein from pea. The native transit peptide was removed and replaced with the transit peptide from the pea small subunit of ribulose-1,5-bisphosphate carboxylase (Rbcs) (A.R. Cashmore, in *Genetic Engineering of Plants*, T. Kosuge, C.P. Meredith, A. Hollaender, Eds. (Plenum Press, New York, 1983) pp. 27-38). Expression of the gene construct was facilitated by the strong CaMV 35S promoter (Odell *et al.*, 1985) and transcriptional termination signals originated from the pea Cab gene (Cashmore, 1984).

The first step of the cloning process was the construction of the pSSTP vector containing a DNA sequence encoding the Rbcs 5'UTR and transit peptide. The DNA fragment containing the required components was retrieved from plasmid pSSNPT (A.R. Cashmore) by first cleaving with the restriction endonuclease *Hind*III. Phenol and

chloroform:isoamyl alcohol extraction and ethanol precipitation in the presence of 0.1 M sodium chloride followed by a 70% ethanol wash were applied after each step of DNA manipulation to inactivate enzymes and to concentrate the DNA. The DNA was collected by centrifugation, dried and redissolved in 10 µl of water. The *Hind*III end was rendered blunt utilizing the Klenow fragment of *E. coli* DNA polymerase I. The reaction consisted of 1 unit of Klenow, 0.1 mM each of dATP, dCTP, dGTP and dTTP, 50 mM Tris-HCl pH7.5, 10 mM magnesium chloride, 5 mM dithiothreitol, and the DNA from the above step, and was incubated at 37°C for 1 hour. After repurification by organic solvent extractions, the DNA was further digested with *Bam*HI in the same manner as for *Hind*III, separating the required DNA fragment from the rest of the pSSNPT plasmid. The *Hind*III-*Bam*HI DNA fragment was gel purified and ligated into the *Sma*I and *Bam*HI sites of pGEM4 (Promega) that had been cleaved and subsequently dephosphorylated by calf intestinal alkaline phosphatase. The purification of DNA was carried out using the standard low melting agarose gel (BRL) and phenol extraction method (Sambrook *et al.*, 1989). DNA was recovered from appropriate low melting agarose slices by heating at 65°C followed by extraction with phenol, prewarmed initially at 37°C, and centrifugation. The phenol extraction was repeated once more. The aqueous DNA layer containing the DNA was then adjusted to 0.1 M sodium chloride and centrifuged for 10 min in a microfuge. The supernatant was then given a chloroform:isoamyl alcohol extraction followed by precipitation in ethanol as described above. The DNA pellet was then collected by centrifugation, washed with 70% ethanol, dried and resuspended in water.

The mature type I LhcIIb Cab coding DNA sequence (pea

AB80), contained in a *Xba*I-*Pst*I DNA fragment, was retrieved by digesting plasmid pDX80 (A.R. Cashmore) with *Xba*I and *Pst*I. The DNA fragment was also gel purified using standard procedures. The isolated DNA fragment was then inserted into the plasmid vector pSSTP via the *Xba*I and PstI sites. Prior to ligation, these sites had been dephosphorylated by adjusting the restriction digestion reaction with 3.5 µl 1 M Tris-HCl, pH 8.0 and adding 0.5 units of calf intestinal alkaline phosphatase. Incubation proceeded for 30 minutes at 37°C and the dephosphorylated vector was then purified by organic solvent extraction followed by ethanol precipitation as above. The plasmid resulting from the ligation was designated pRBCS-CAB.

The resulting Rbcs-Cab chimeric gene was then fused to the 35S CaMV constitutive promoter by inserting a gel-purified *Eco*RI-*Hind*III fragment carrying the 35S CaMV promoter from plasmid pCAMV (A.R. Cashmore) into the *Eco*RI-*Asp*718 sites of pRBCS-CAB. The corresponding HindIII and *Asp*718 restriction sites were made blunt using the Klenow fragment of DNA polymerase I. The 35S CaMV-Rbcs-Cab construct was then transferred as an *Eco*RI-*Pvu*II DNA fragment to the *Bam*HI site of the binary vector pEND4K (Figure 1) (Klee et al., 1985). All of the restriction enzyme-generated ends were made blunt by Klenow in this step.

All ligation steps were carried out at 15°C overnight using T4 DNA ligase from various suppliers. All steps of the gene construction process were carried out using the standard $CaCl_2$ bacterial transformation protocol and the *E. coli* host strain HB101. All recombinant plasmids were propagated in HB101 and isolated using standard techniques.

The resolution of DNA fragments was facilitated by using standard agarose and polyacrylamide gel electrophoresis techniques.

Construction of a CaMV-Bce44B-NOS Transgene for Expression in Plants

Transgenic plants (*Brassica napus*) were produced with a transgene construct for protein transport consisting of DNA sequences encoding the Bce44B protein and the 5' untranslated region immediately upstream of the Bce44B coding sequence. Expression of the transgene construct was facilitated by the strong cauliflower mosaic virus 35S promoter (J.T. Odell, F. Nagy, N.H. Chua 1985, *Nature* **313**, 810) and the transcriptional termination signal originated from the gene encoding nopaline synthase (NOS) found in *Agrobacterium* Ti plasmids or their derivatives such as pBI101 (Clontech).

The initial step of the transgene cloning process was the construction of a vector containing the DNA sequence encoding the 35S CAMV promoter to facilitate the cloning strategy for the final 35SCAMV-Bce44B-NOS transgene. The DNA cloning procedures used followed standard procedures. The 35S CAMV constitutive promoter was retrieved as an *Eco*RI-*Hind*III DNA fragment (approximately 450 base pairs in length) from pCAMV (A.R. Cashmore). The *Hind*III restriction site was converted to a blunt end by using Klenow. The DNA fragment was then inserted into the *Eco*RI and *Sma*I sites of pGEM4 (Promega). This new vector, designated pCAMV2, was used to fuse the 35S CAMV promoter to the Bce44B coding region. The fusion was achieved by retrieving Bce44B as an 1,193 base pair EcoRI-*Hind*III DNA fragment from pBce44B and inserting the DNA fragment into the *Xba*I-*Hind*III sites of

pCAMV2. The *Eco*RI and *Xba*I sites were made into blunt end sites using Klenow. The resulting vector was designated pCAMV-Bce44B. The 3' transcription termination sequence from the NOS gene was then added to the pCAMV-Bce44B by inserting an *Eco*RI-*Hind*III DNA fragment (approximately 260 base pairs in length) into the *Not*I-*Hind*III sites of pCAMV-Bce44B. The *Eco*RI and *Not*I sites were made blunt using Klenow. The NOS transcription termination sequence was retrieved from the pBI121 binary vector (Clontech) as a *Sac*I-*Eco*RI DNA fragment (approximately 260 base pairs in length). In order to obtain the appropriate restriction sites to insert the NOS transcription termination sequence into pCAMV-Bce44B, the *Sac*I-*Eco*RI DNA fragment was first inserted into the *Sac*I-*Sma*I sites of pGEM4 (this vector was designated pNOS for reference purposes) and then retrieved back out as the *Eco*RI-*Hind*III DNA fragment described above. The resulting final transgene-containing vector was designated pCAMV-Bce44B-NOS. The 35SCAMV-Bce44B-NOS transgene was then transferred as a *Bam*HI DNA fragment from pCAMV-Bce44B-NOS to the *Bam*HI site of the binary vector pEND4K (H. Klee, M.F. Yanofsky, E.W. Nester 1985, *Biotechnology* **3**, 637; R.B. Horsch et al. 1985, *Science* **227**, 181; M. Holsters et al. 1987, *Mol. Gen. Genet.* **163**, 181). The transgene-containing *Agrobacterium* binary vector was designated pEND4K-CAMV-Bce44B-NOS (Figure 2).

All ligation steps were carried out at 15°C overnight using T4 DNA ligase from various suppliers. All steps of the gene construction process were carried out using the standard $CaCl_2$ bacterial transformation protocol and the *E. coli* host strain HB101. All recombinant plasmids were propagated in HB101 and isolated using standard techniques.

The resolution of DNA fragments was facilitated by using standard agarose and polyacrylamide gel electrophoresis techniques.

Example 3. Transformation of *Agrobacterium* strains and growth conditions

Introduction of the pEND4K-CAMV-Rbcs-Cab and pEND4K-CAMV-Bce44B-NOS transgene constructs into *Agrobacterium tumafaciens* was carried out using the freeze thaw method. Competent *Agrobacterium* (strains such as LBA4404 or GV3101) cells were obtained by inoculating 50 mL of LB broth containing the appropriate antibiotics (50μg/mL rifampicin for LBA4404 or 100 mg/ml gentamycin and 150 mg/ml rifampicin for GV3101) with 500 μL of an overnight culture, incubating them at 28°C with vigorous shaking until the optical density at 650 nm was 0.7. Cells were harvested by centrifugation at 2000g x 5min at 4°C, washed in ice cold 0.1M $CaCl_2$ and finally resuspended in 1 mL of ice cold 20 mM $CaCl_2$. A 150 μL aliquot of competent LBA4404 or GV3101 cells was removed and mixed with 1 μg of plasmid DNA in a microfuge tube, and immediately frozen in liquid nitrogen. The cells were incubated at 37°C in a water bath or thermostat block for 5 min, 1 mL of LB broth was added and the mixture incubated at 28°C with shaking for 3h. After this period of time, cells were recovered by centrifuging at 2000 xg for 5 min and resuspended in 100 μL of LB. Cells were plated on LB plates containing appropriate levels of antibiotics (100 μg/mL kanamycin and 50 μg/mL rifampicin for the LBA4404 strain or 50 μg/mL kanamycin, 150 μg/mL rifampicin and 100 μg/mL gentamycin for the GV3101 strain) and incubated for 2-4 days at 28°C. Kanamycin-resistant colonies growing on the plates were selected and the presence of the pEND4K-CAMV-Bce44B-NOS plasmid confirmed by restriction endonuclease digestion

analysis and DNA blot hybridization analysis of plasmid preparations obtained from single colonies as follows. Three mL of LB broth containing appropriate levels of antibiotics as described above for the different *Agrobacterium* strains was inoculated with a single colony growing on selective antibiotic-containing LB plates and incubated from overnight to 2 days at 28°C with shaking. A 1.5 mL sample of these cultures was placed in a microfuge tube and centrifuge for 30 sec in a microfuge to collect the cells. The cell pellet was resuspended in 0.1 mL of GTE solution (50 mM glucose, 10 mM Na_2EDTA, 25 mM Tris-HCl pH 8.0) and 4 mg/ml of lysozyme, and incubated at room temperature for 10 min. Phenol (30 μL), previously equilibrated with 2 vols of 1% (w/v) SDS, 0.2N NaOH, was added. The mixture was vortexed gently until viscous and incubated at room temperature for 10 min. The lysed cells were neutralized with 3M sodium acetate pH 4.8 (150 μL) and incubated at -20°C for 15 min. The mixture was centrifuged for 3 minutes in a microfuge and the supernatant transferred to a fresh microfuge tube. Two volumes of ethanol were added and mixed, and the mixture was incubated at -80°C for 15 minutes. The mixture was then centrifuged for 3 minutes and the DNA pellet resuspended in 90 μL of water. Ten μL of 3M sodium acetate pH 7.0 were added, followed by an equal volume of phenol/chloroform and mixed by vortexing. After centrifuging for 5 min in a microfuge, the supernatant was transferred to a fresh tube and the DNA precipitated by adding 2 volumes of 100% ethanol. After centrifugation for 10 min, the pellet was washed with 70% ethanol dried and resuspended in 50 μL of TE (10 mM Tris-HCl pH 8.0, 1 mM Na_2EDTA).

Integrity of the pEND4K-CAMV-Rbcs-Cab and pEND4K-CAMV-Bce44B-NOS plasmids in *Agrobacterium* was verified by

restriction endonuclease digestion analysis and Southern DNA blot hybridization analysis of the plasmid isolated as described above and in Molecular Cloning: A Laboratory Manual (Sambrook et al. 1989, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). The *Agrobacterium* colonies containing intact pEND4K-CAMV-Rbcs-Cab and pEND4K-CAMV-Bce44B-NOS vectors, respectively, were selected for plant transformation.

Example 4. *Agrobacterium*-mediated *in planta* transformation

Approximately 6 weeks after germination, plants were ready for transformation. One apical shoot on each plant was severed with a razor blade and the wound was inoculated with 100 µl of an overnight *Agrobacterium* culture containing the desired gene construct. Plants were not watered for the next 2-3 days to allow efficient infiltration of the bacterial suspension. After one week, a number of secondary shoots appeared. These were removed and fresh inoculum was applied to the wound sites. Third and fourth inoculations were performed similarly at one week intervals. The plants, designated T1, were grown to maturity and T2 seeds were bulk harvested.

Example 5. *Agrobacterium*-mediated *in planta* transformation using rock wool

Another variation of the transformation protocol was used to transform *Brassica napus* cv. Quantum. This involved using rock wool (Agrodynamics, East Brunswick, NJ), which is a substrate made from volcanic rock that has been converted into mineral wool. A 1 cm^2 piece of the wool was saturated with an overnight *Agrobacterium* culture and placed on top of the apical shoot which had been sliced off with a razor

blade. The plug was then remoistened with the *Agrobacterium* culture once on each of the subsequent two days. The plug then sat on the wound until it had dried and fallen off, or was removed during the next round of inoculations.

Example 6. Transformation of Corn and Wheat

Corn (*Zea mays*) and wheat (*Triticum* sp.) were grown and prepared for inoculation under conditions similar to those for *Brassica*, lettuce and flax. *Agrobacterium* (LB4404) containing the transgene was grown overnight in LB media (29°C, 250 rpm), centrifuged and resuspended in M9 minimal media containing 0.2 mM acetosyringone (3',5'-Dimethoxy-4'-hydroxyacetophenone) (5X initial culture volume) and allowed to continue growing at 29°C with shaking overnight. It has been shown that acetosyringone is useful to induce *Agrobacteria* for better transformation of plants. (Escudero and Hohn, 1997; Escudero, *et al.*, 1995). The culture was centrifuged, resuspended in 10 mM $MgSO_4$ (same volume) and one of the following methods used to apply the culture:

a. Plants were injected using a 27 gauge needle approximately 1" from the base of the stalk (or just above where the stalk becomes hollow in the older plants). Bacterial suspension was injected until drops were seen emerging from the top of the stalk.

b. Plant "tubes" (formed by leaves) were filled with bacterial suspension from the top using a 22 gauge needle without causing damage to the plant. This method is preferable for use in corn due to the more compact form of the wheat "tube".

c. Plants were wounded using a 26 gauge needle from the base of the stalk up to the point where the latest leaves were emerging. Plants were then

sprayed with bacterial suspension using a standard pump spray bottle.

d. Plants were sprayed with bacterial suspension as in c, but without wounding the plant.

Each method was repeated weekly for a total of three application of *Agrobacterium* culture. Plants were then allowed to grow and produce seed. Harvested seed was selected on kanamycin.

Example 7. Analysis of T2 progeny

The progeny seeds (T2) collected from primary transformed T1 plants were surface sterilized with 75% ethanol for 15 sec, 1% Na hypochlorite + 1/1500 dilution of Tween20 for 60 min, then washed twice with sterile distilled water. The seeds were plated on solid 0.5 x MS medium (Sigma) supplemented with 75 µg/ml kanamycin (Sigma). After a two-night cold treatment at 4°C to synchronize germination, the boxes were transferred to a growth chamber at 22°C, 16 hour photoperiod, 100 µE m^2/s light intensity. Three weeks after germination, kanamycin-resistant T2 seedlings were transplanted into 6 inch pots of soil and grown to maturity. T3 seeds were individually harvested.

Example 8. DNA isolation and Southern Analysis

DNA was isolated from individual T2 plants by the method of *Landry et al.* (1987). Fresh leaves (10-30g) were blended with a Polytron homogenizer in 160 ml ice-cold H buffer (4 mM spermidine, 1 mM spermine, 10 mM EDTA, 10 mM Tris, 80 mM KCl , 1 mM PMSF, 50 mM sucrose, 0.2% 2-mercaptoethanol, pH 9.5) at medium rpm. The homogenate was filtered through four layers of Miracloth (Calbiochem) into a 260 ml centrifuge bottle. After centrifugation at 2,000 x g, 4°C, for 20 min, the supernatant was discarded and the

pellet resuspended in 40 ml of ice cold HT buffer (1 x H buffer, 0.5% Triton X-100). The suspension was again centrifuged at 2,000 x g, 4°C, for 10 min in a 50 ml polycarbonate tube and the supernatant discarded. Resuspension of the pellet in HT buffer and centrifugation was repeated three times until the pellet of nuclei was grey. The pellet was then resuspended in 7.5 ml HT buffer and 7.5 ml lysis buffer (100 mM Tris, 40 mM EDTA, 2% Na-sarcosyl, pH 9.5) was added. Immediately, 16.46 g of CsCl were added and the tubes were incubated at 55-60°C for 1 hour with occasional inversions. After the CsCl had dissolved, the tubes were centrifuged at 15,000 rpm for 30 min, 15°C. The supernatant was filtered through 1 layer of Miracloth into two 15-ml polycarbonate, screw-capped ultracentrifuge tubes (Beckman) each containing 400 µl of ethidium bromide (10 mg/ml). The samples were ultracentrifuged at 45,000 rpm for 48 hours, 15°C in Ti70.1 fixed angle rotor (Beckman). DNA was recovered using standard procedures (Maniatis *et al.*, 1982) and resuspended in sterile water at a final concentration of 0.5 µg/ml.

DNA samples were cut with restrictions enzymes (*Eco*RI, *Hind*III or *Sal*I), in accordance with the buffers and protocols provided by the manufacturer of each particular enzyme. Restriction enzyme was added to give approximately 100 units per µg of DNA and the reaction mixture was adjusted to the appropriate final volume with water. The final volumes were usually 200 µl and contained 1µg of genomic DNA. Digestions were carried out for 12 hours at the appropriate temperature. Digested DNA was concentrated by precipitation in two volumes of 100% ethanol in the presence of 0.1M sodium chloride. The DNA precipitates were collected by centrifugation, washed once with 70% ethanol,

dried and redissolved in water. The digested DNA was resolved by electrophoresis on a 0.7% agarose gel and transferred to a nitrocellulose filter (Schleicher and Schuell) using standard procedures. The probe (a 2 kb NPTII fragment) was labeled with α-[^{32}P]dCTP (ICN) using the Nick Translation Kit from Gibco BRL. Hybridization was carried out at 60°C in 5 x SSC, 5 x Denhardt's, 50 mM sodium phosphate pH 6.5, 2.5 mM EDTA pH 8.0, 5% dextran sulfate, and 100 µg/ml denatured salmon sperm DNA. Filters were washed once with 5 x SSC/0.1%SDS for 15 min. at room temperature, twice with 2 x SSC/0.1%SDS at 50°C, followed by a 0.2 x SSC/0.1% SDS wash at 50°C, and then were exposed to Kodak XAR-5 film, between CRONEX Intensifying screens, for several days at -70°C.

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EQUIVALENTS

While this invention has been particlarly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the spirit and scope of the invention as defined by the appended claims. Those skilled in the art will recognize or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described specifically herein. Such equivalents are intended to be encompassed in the scope of the claims.

CLAIMS

What is claimed is:

1. A method of delivering isolated DNA to the cells of a plant comprising the steps of:
   a) wounding the plant; and
   b) applying the isolated DNA to the wound under conditions whereby additional DNA can be applied to the wound site over a period of two or more days to enhance transformation efficiency.

2. The method of Claim 1 wherein the plant is selected from the group consisting of lettuce, *Brassica* sp., or flax, corn, wheat, tomato, soybean, basil, pea, bean, alfalfa, and petunia.

3. The method of Claim 2 wherein the isolated DNA is delivered in a transformation-competent *Agrobacterium* cell.

4. The method of Claim 1 wherein the wounding is done by cutting the plant at its base.

5. The method of Claim 1 wherein the wounding is done by dissection, puncturing, cutting, electroporation, bolistics or microparticle bombardment, or two or more of these techniques.

6. The method of Claim 1 wherein an abrasive composition is applied to the wound prior to or at the time the DNA is applied to the wound.

7. The method of Claim 6 wherein the abrasive composition

is rock wool.

8. The method of Claim 1 wherein the wounding is done by a compound which degrades or dissolves the plant cell wall or cuticle.

9. The method of Claim 1 wherein a compound selected from the group consisting of phomalide, soaps and detergents is added to the wound site.

10. A transformed plant produced by the methods of Claim 1.

11. A portion of a transformed plant produced by the methods of Claim 1.

12. The portion of Claim 11 selected from the group consisting of leaves, stems, roots, flowers, seeds, meristems, tissues, protoplasts, embryos, pollen, ovules, explants, and cells.

13. A transgenic plant produced from the portion of Claim 12.

14. Seed of the plant of Claim 10.

15. A plant produced from the seed of the plant of Claim 14.

16. A method for transforming a plant with isolated DNA comprising:

a) preparing a transformation competent *Agrobacterium* sp. containing a Ti plasmid, wherein the DNA is contained in the plasmid;

c) maintaining the *Agrobacterium* sp. in a cultivation

medium;

d) wounding the plant;

e) applying to the wound, the *Agrobacterium* sp. in the cultivation medium with a carrier which also assists in the entry of the *Agrobacterium* sp. and which further permits the *Agrobacterium* sp. in culture solution to be replaced with additional fresh *Agrobacterium* sp.;

f) maintaining the *Agrobacterium* sp. in the wound until the DNA is introduced into the plant and transformation of plant cells occurs;

g) selecting for growing portions of the plant which are transformed;

thereby producing a transformed plant or portion of a plant.

17. The method of Claim 16 wherein the plant is selected from the group consisting of lettuce, *Brassica* sp., or flax, corn, wheat, tomato, soybean, basil, pea, bean, alfalfa, and petunia.

18. The method of Claim 16 wherein the carrier is an abrasive substance.

19. The method of Claim 16 wherein the carrier is rock wool.

20. The method of Claim 16 wherein the carrier is a wetting agent.

21. The method of Claim 16 wherein the wounding is done by a compound which degrades or dissolves the plant cell wall or cuticle.

22. The method of Claim 16 wherein a compound selected from the group consisting of phomalide, soaps and detergents is added to the wound site.

23. A transformed plant produced by the method of Claim 16.

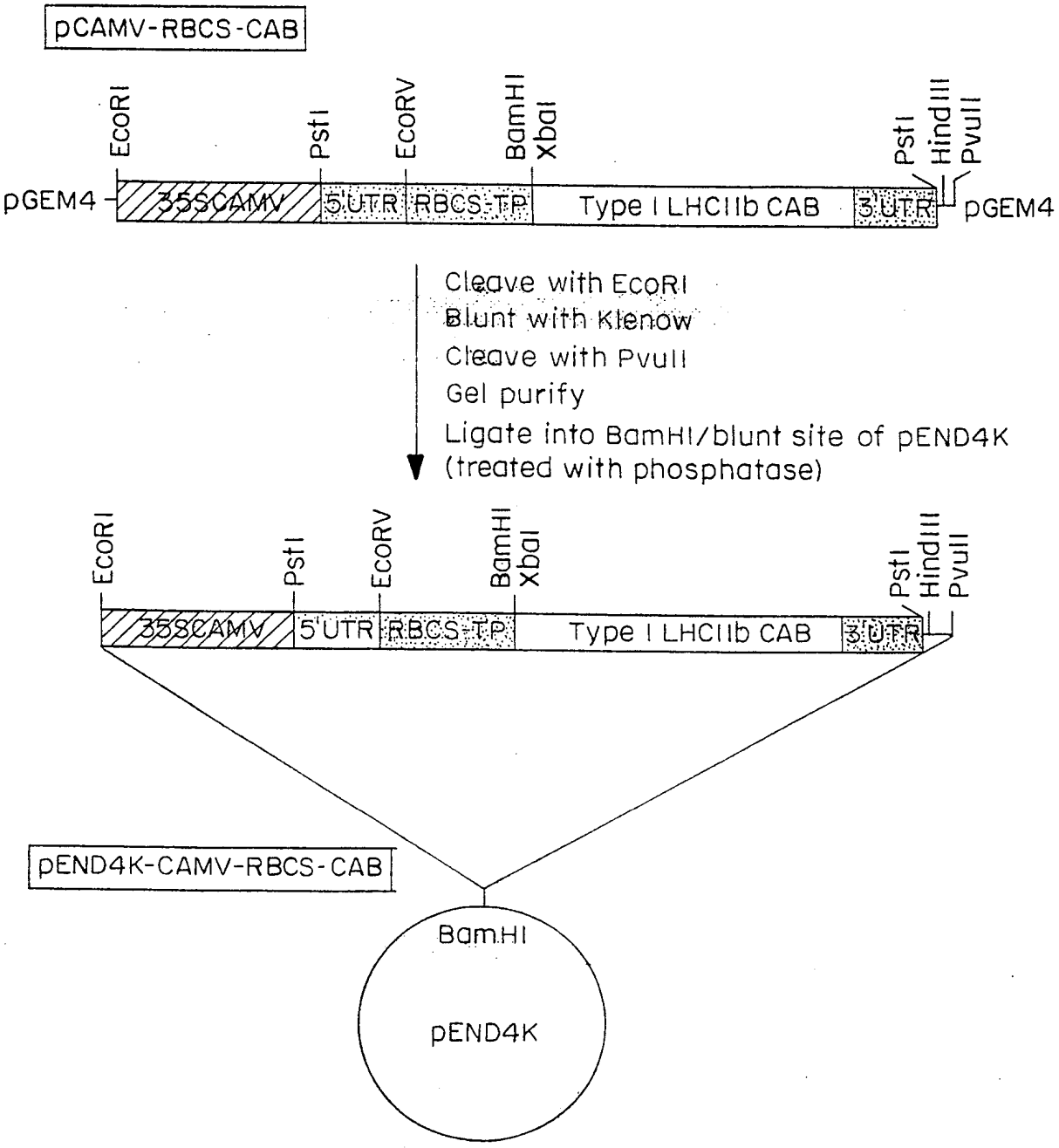
24. A transformed portion of a plant produced by the method of Claim 16.

25. The portion of Claim 24 selected from the group consisting of leaves, stems, roots, flowers, seeds, meristems, tissues, protoplasts, embryos, pollen, ovules, explants, and cells.

26. A transgenic plant produced from the portion of Claim 25.

27. Seed of the plant of Claim 26.

28. A plant produced from the seed of the plant of Claim 27.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PstI
EcoRV
BamHI
XbaI
PstI
HindIII
PvuII
pGEM4
35SCAMV
5'UTR
RBCS-TP
Type I LHCIIb CAB
3'UTR
pGEM4
Cleave with EcoRI
Blunt with Klenow
Cleave with PvuII
Gel purify
Ligate into BamHI/blunt site of pEND4K
(treated with phosphatase)
EcoRI
PstI
EcoRV
BamHI
XbaI
PstI
HindIII
PvuII
35SCAMV
5'UTR
RBCS-TP
Type I LHCIIb CAB
3'UTR
pEND4K-CAMV-RBCS-CAB
BamHI
pEND4K

FIG. 1

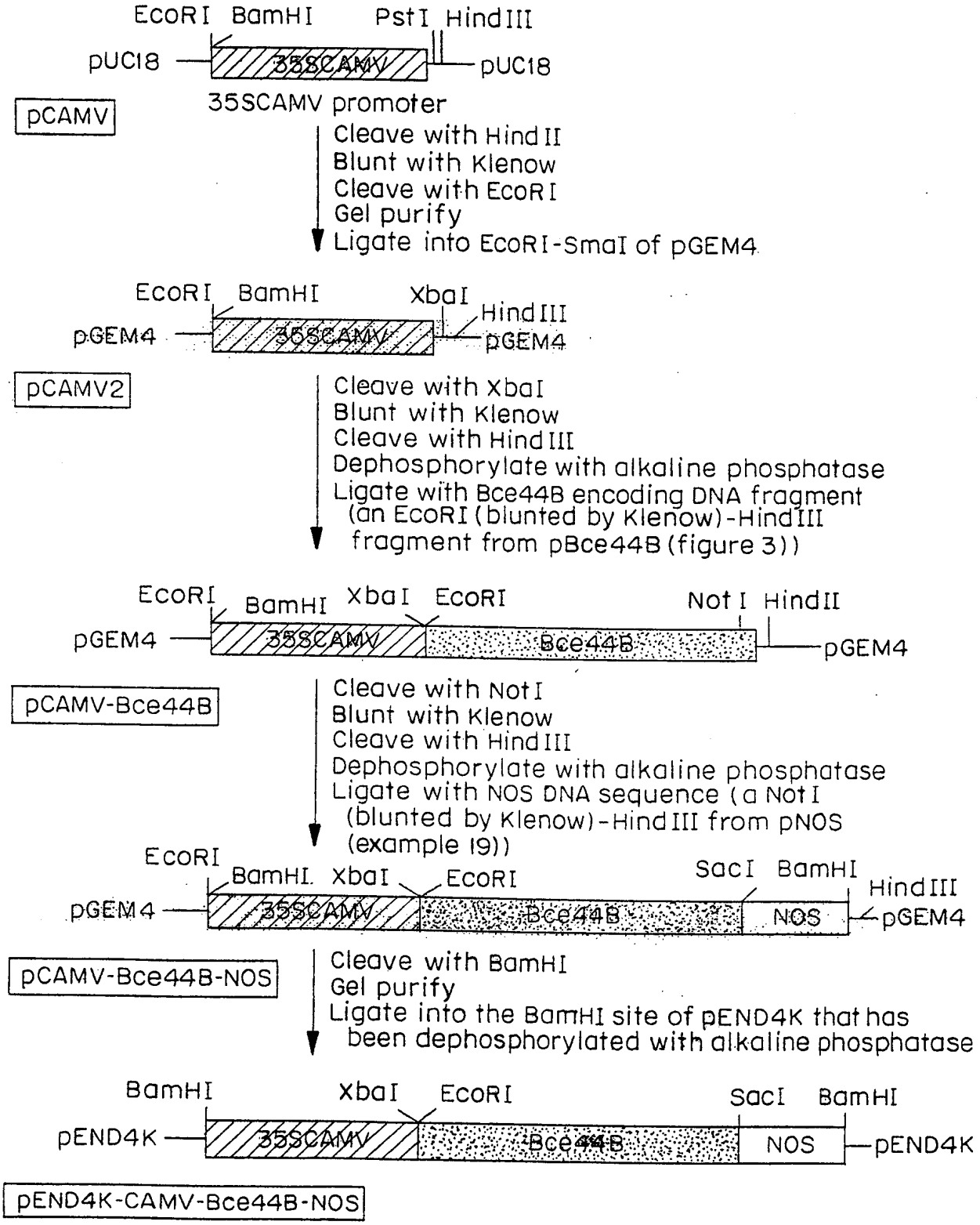
EcoRI BamHI PstI HindIII
pUC18
35SCAMV
pUC18
35SCAMV promoter
pCAMV
Cleave with HindII
Blunt with Klenow
Cleave with EcoRI
Gel purify
Ligate into EcoRI-SmaI of pGEM4
EcoRI BamHI XbaI HindIII
pGEM4
35SCAMV
pGEM4
pCAMV2
Cleave with XbaI
Blunt with Klenow
Cleave with HindIII
Dephosphorylate with alkaline phosphatase
Ligate with Bce44B encoding DNA fragment
(an EcoRI (blunted by Klenow)-HindIII
fragment from pBce44B (figure 3))
EcoRI BamHI XbaI EcoRI NotI HindII
pGEM4
35SCAMV
Bce44B
pGEM4
pCAMV-Bce44B
Cleave with NotI
Blunt with Klenow
Cleave with HindIII
Dephosphorylate with alkaline phosphatase
Ligate with NOS DNA sequence (a NotI
(blunted by Klenow)-HindIII from pNOS
(example 19))
EcoRI BamHI XbaI EcoRI SacI BamHI HindIII
pGEM4
35SCAMV
Bce44B
NOS
pGEM4
pCAMV-Bce44B-NOS
Cleave with BamHI
Gel purify
Ligate into the BamHI site of pEND4K that has
been dephosphorylated with alkaline phosphatase
BamHI XbaI EcoRI SacI BamHI
pEND4K
35SCAMV
Bce44B
NOS
pEND4K
pEND4K-CAMV-Bce44B-NOS

FIG. 2

INTERNATIONAL SEARCH REPORT

Int. ational Application No
PCT/CA 98/00859

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/82 A01H1/00 A01H5/00 A01H5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KATAVIC, V. ET AL.: "In planta transformation of Arabidopsis thaliana" MOLECULAR & GENERAL GENETICS, vol. 245, 1994, pages 363-370, XP002085791 cited in the application see page 364 - page 365 'Materials and methds' and 'Results - In planta transformation' see figure 1 ---	1,4,5, 10-16, 23-28
X	CHANG, S.S. ET AL.: "Stable genetic transformation of Arabidopsis thaliana by Agrobacterium inoculation in planta" PLANT JOURNAL, vol. 5, no. 4, 1994, pages 551-558, XP002085792 see page 556 - page 558 'Discussion' and 'Experimental procedures' --- -/--	1,4-6, 10-16, 23-28

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 November 1998	09/12/1998

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

In ational Application No
PCT/CA 98/00859

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication.where appropriate. of the relevant passages	Relevant to claim No.
X	WO 95 15678 A (THE TEXAS A &M UNIVERSITY SYSTEM) 15 June 1995 see page 5, line 2 - page 6, line 21 see page 9, line 10 - page 10, line 17 see claims 1,7-9,26,28-30; example 1	1,4,5, 10-15, 23-28
X Y	WO 92 09696 A (PLANT GENETIC SYSTEMS, N.V.) 11 June 1992 see page 2, line 32 - page 5, line 2 see page 9, line 21 - page 11, line 8 see claims 1,3-9,1,13,14,15; examples 1,3,11	1,2,4-6, 8,10-15 16,17, 21,23-28
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